

# OIE Standards on Rabies Diagnostics

Changchun Tu



OIE Reference Laboratory for Rabies  
Changchun Veterinary Research Institute, China

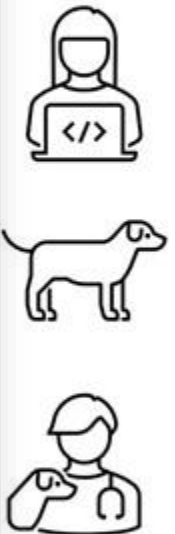
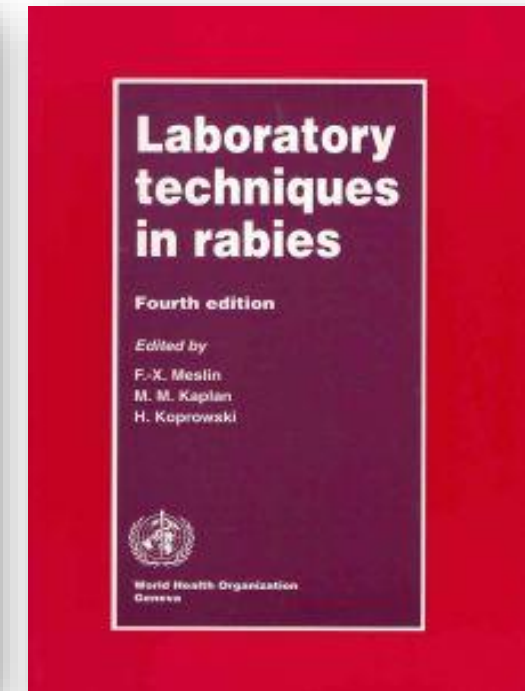
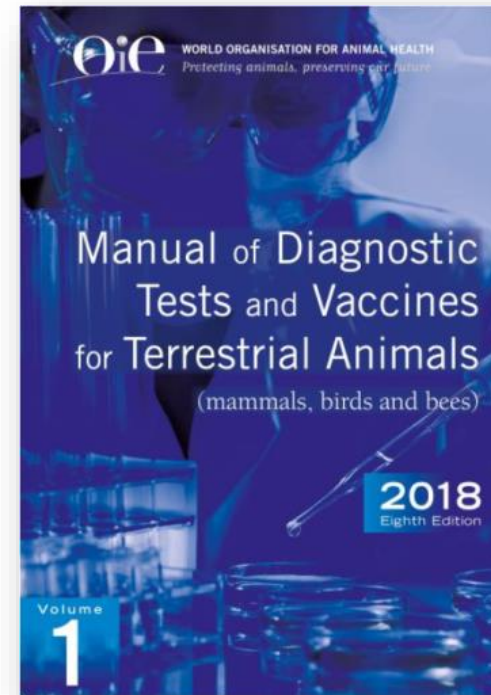
OIE SEA Sub-Regional Virtual Workshop on  
OIE international standards supporting self-declaration  
of freedom from rabies,  
and endorsement of official control programme  
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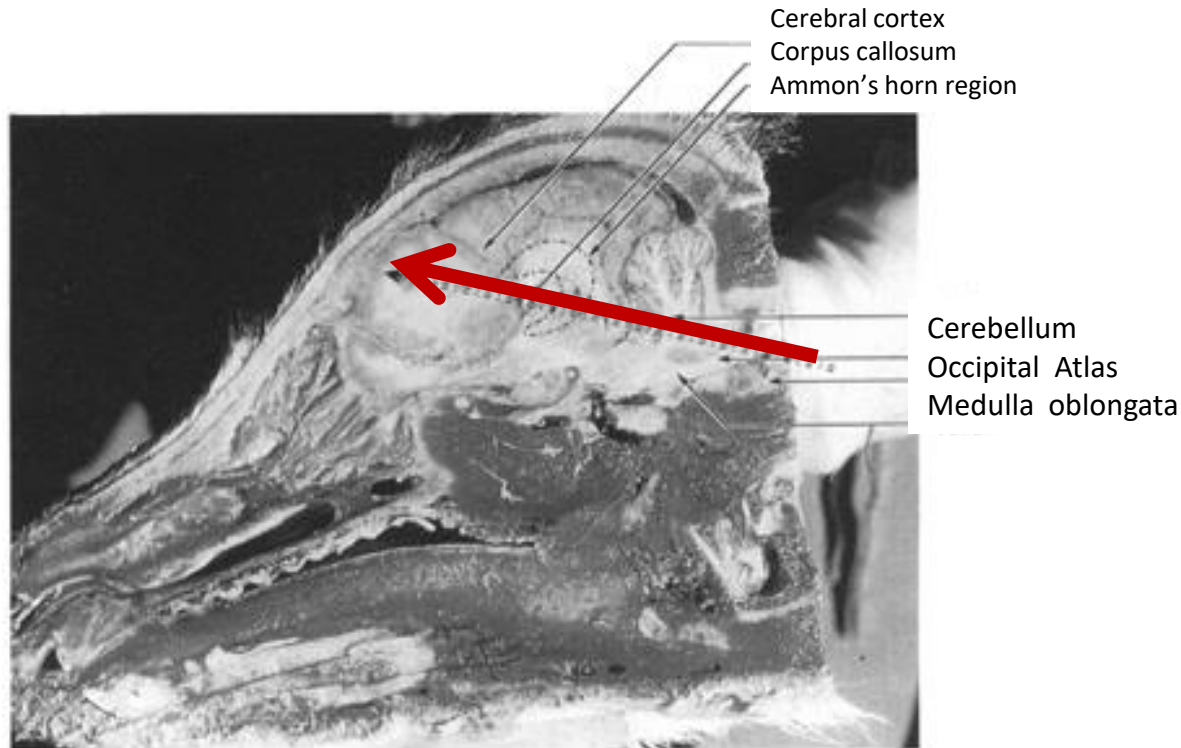
# Rabies Diagnosis

1. Sample collection
2. Shipment
3. Laboratory tests
  - (1) Detection of viral antigen
    - a. **DFA**
    - b. dRIT
  - (2) Detection of live virus
    - a. Cell culture test (RTCIT)
    - b. Mouse inoculation test (MIT)
  - (3) Molecular techniques
    - a. **Conventional RT-PCR**
    - b. **real-time PCR**
4. Serology tests
  - (1) virus neutralisation: FAVN and RFFIT
  - (2) **antibody ELISA**



# 1.1 Collection of Brain Samples

Straw method: occipital foramen route for brain sampling



Insert the straw into the occipital foramen towards one eye. Samples are taken from the medulla oblongata, the base of the cerebellum, the hippocampus and the cerebral cortex.



# Collection of brain samples

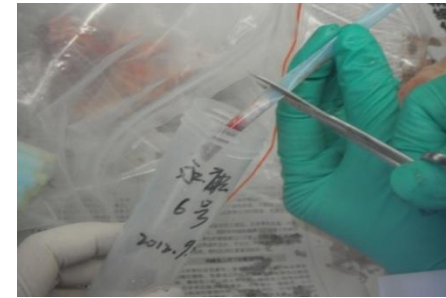
## Occipital foramen route for brain sampling



Expose the occipital foramen



Insert plastic straw



1. Transfer the tissues into tubes, and transit to the laboratory in ice container
2. Refrigeration during transit is not needed If the sample is in glycerol



# 1.2 Shipment of Samples



Journal of Virological Methods  
Volume 140, Issues 1–2, March 2007, Pages 174–182



## Use of filter paper (FTA®) technology for sampling, recovery and molecular characterisation of rabies viruses



The stability of viral RNA and the inactivation of infectivity make the FTA® cards useful for the storage, transport, collection and subsequent molecular analysis of viral rabies RNA.

- Impregnated with chemical formula that lyses cell and denature protein upon contact.
- The chemical formula can inactivate virus after a 2 h contact with FTA® cards.
- Widely used for bacteria, viruses, variety of tissues in any form
- Protecting NA and denaturing protein make FTA useful for PCR, **not for antigen detection (such as DFA, etc).**
- RNA could be stored 35-48 days on FTA® cards.
- International shipment is possible without import permit.



# 1.3 Laboratory tests

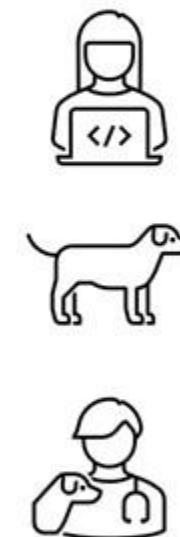
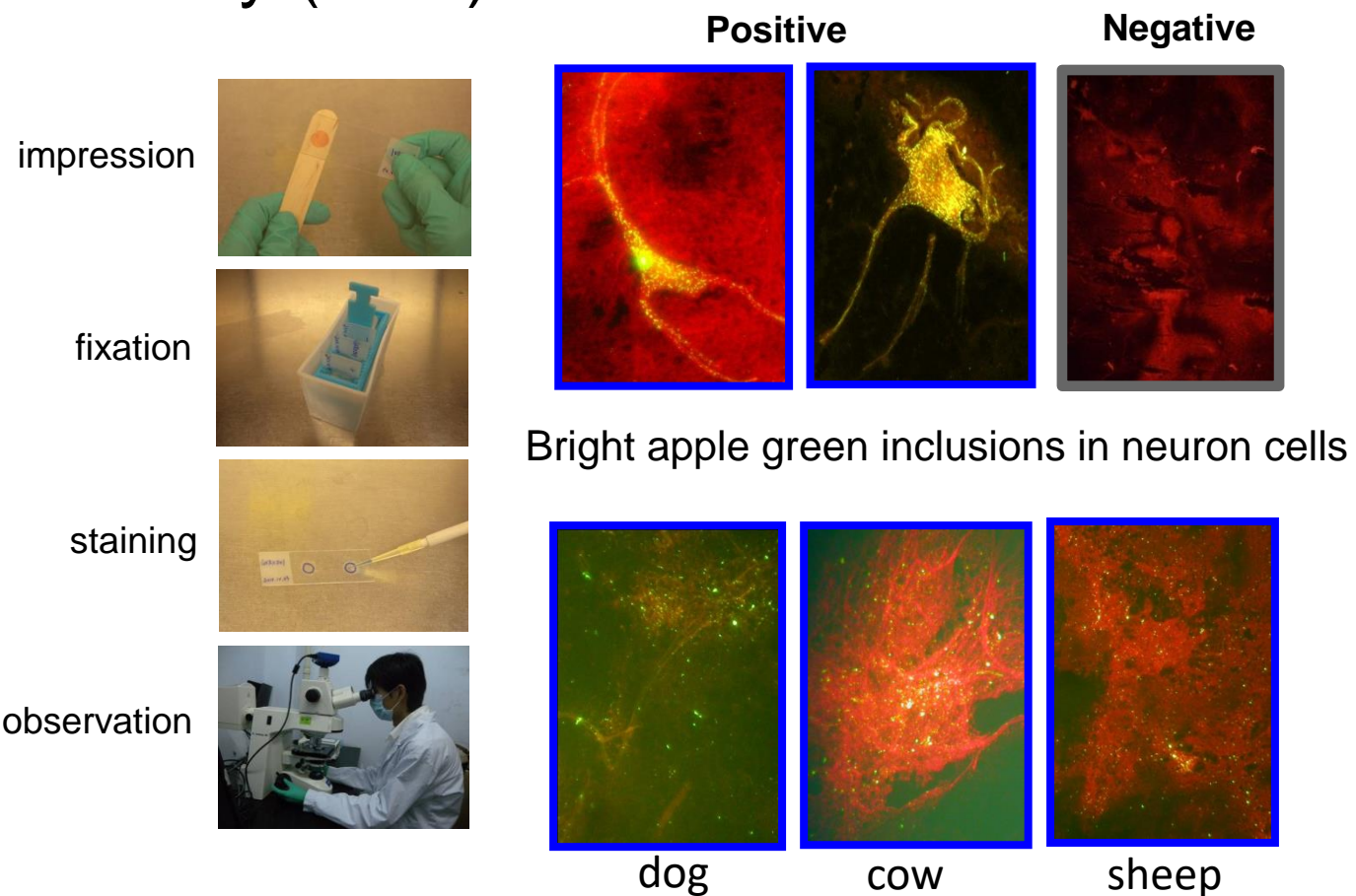
Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification						
DFA (antigen detection)	+++	n/a	+++	+++	+++	n/a
dRIT (antigen detection)	+++	n/a	+++	+++	+++	n/a
ELISA (antigen detection)	+	n/a	+	+	+	n/a
Cell culture (virus isolation)	+	n/a	+++	+++	+++	n/a
MIT (virus isolation)	n/a	n/a	+	+	+	n/a
Conventional RT-PCR (RNA detection)	+++	n/a	+++	+++	+++	n/a
Real-time RT-PCR (RNA detection)	+++	n/a	+++	+++	+++	n/a



## 1.3.1 Immunochemical identification of rabies virus antigen

### i) Direct fluorescent antibody (DFA) Test

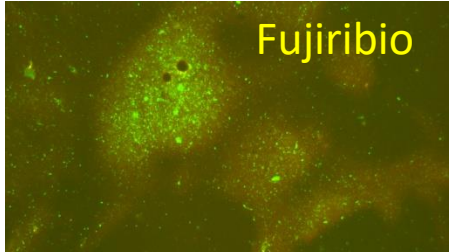
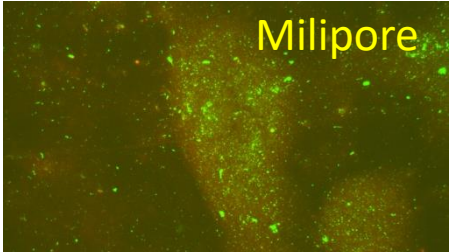
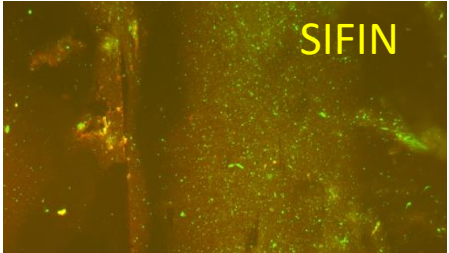
- Golden standard of OIE and WHO
- suitable for brain tissue; cell monolayers
- simple with high sensitivity and specificity
- 98-100% accuracy
- Requisite for rabies laboratory
- National Standard of China (GB/T18639-2002)
- **Need fluorescent microscope, trained technicians**





# i) Direct fluorescent antibody (DFA) Test

Comparison of various anti-rabies conjugates

RABV			
	Fujiribio	Milipore	SIFIN
	Fujiribio (USA)	Milipore (UK)	SIFIN (Germany)
RABV	++++	++++	++++
LBV	+	++	++++
MOKV	+	++++	++++
DUVV	+	++	+
EBLV-1	+	+++	+++
EBLV-2	++	+++	+
ABLV	+++	++++	++

Scores: 4 +++++: very bright green fluorescence  
 3 +++ bright green fluorescence  
 2 ++ dull green fluorescence  
 1 + dim but detectable green fluorescence





## ii) Direct Rapid Immunohistochemistry Test (dRIT)

- Has the same specificity and sensitivity as FAT
- Used as an alternative to FAT
- Use only light, not fluorescent, microscope
- Suitable for field work

Positive results based on the presence of magenta inclusions on a blue neuronal background.

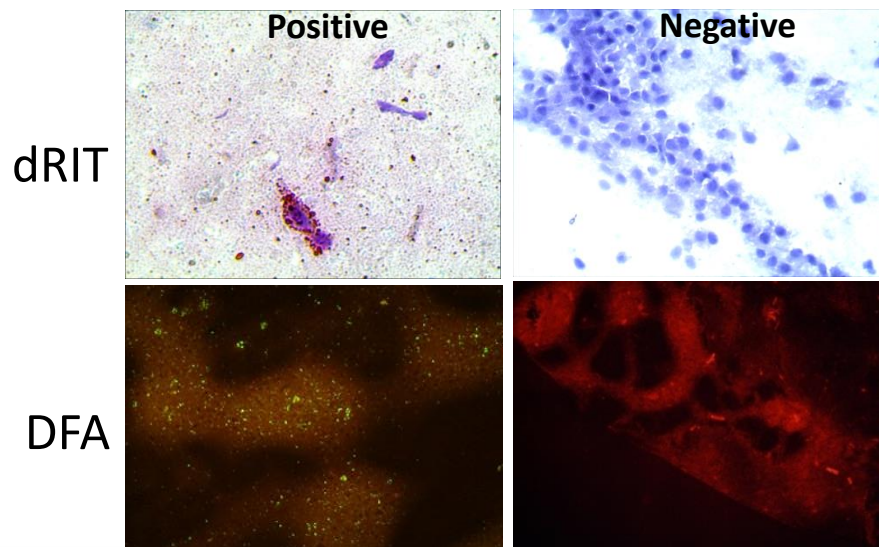


Table 2. Number of Tanzanian brain samples processed by dRIT and DFA for different animal species\*

Species	No. brains examined†
Domestic dog	73 (39)
Domestic cat	7 (3)
Cow	8 (7)
Goat	6 (5)
Livestock‡	1 (1)
Aardwolf ( <i>Proteles cristatus</i> )	1
African civet ( <i>Civettictis civetta</i> )	2
Banded mongoose ( <i>Mungos mungo</i> )	2
Slender mongoose ( <i>Herpestes sanguineus</i> )	3
Dwarf mongoose ( <i>Helogale parvula</i> )	2
White-tailed mongoose ( <i>Ichneumia albicauda</i> )	8 (1)
Mongoose‡	2
Black-backed jackal ( <i>Canis mesomelas</i> )	3
Bat-eared fox ( <i>Otocyon megalotis</i> )	8
Black-backed jackal/bat-eared fox‡	2 (1)
Cheetah ( <i>Acinonyx jubatus</i> )	3
Small-spotted genet ( <i>Genetta genetta</i> )	7 (1)
Lion ( <i>Panthera leo</i> )	6
Serval ( <i>Felis serval</i> )	1
Spotted hyena ( <i>Crocuta crocuta</i> )	12 (1)
Striped hyena ( <i>Hyaena hyaena</i> )	1
Zorilla ( <i>Ictonyx striatus</i> )	1
Total domestic	95 (55)
Total wildlife	64 (4)
Total	159 (59)

\*dRIT, direct immunohistochemical test; DFA, direct fluorescent-antibody assay.

†The number of rabies-positive samples is shown in brackets.

‡Species not definitively identified.

(Lembo, et al. Evaluation of a Direct, Rapid Immunohistochemical Test for Rabies Diagnosis. *Emerg Infect Dis*, 2006)



# ii) Direct Rapid Immunohistochemistry Test (dRIT)

~100% coincident with DFA

## 直接快速免疫组化法在我国狂犬病诊断中的初步应用

陶晓燕, Michael Niezgoda, 杜加亮, 李浩, 王晚元, 黄莹, 康洋, 曹雷, 唐金, 袁国栋

**【摘要】目的** 评价诊断狂犬病的新方法—直接快速免疫组化法 (Direct Rapid Immunohistochemical Test, dRIT) 在我国狂犬病实验室检测工作中的应用推广价值。方法 采用美国疾病预防控制中心 (CDC) 狂犬病实验室建立的欧洲狂犬病抗原的 dRIT 法与目前常用狂犬病诊断方法直接荧光抗体染色 (DFA) 和反转录聚合酶链式反应 (Reverse Transcription Polymerase Chain Reaction, RT-PCR) 检测犬脑组织 (脑干、脑核、小脑、丘脑) 及海马体 4 个部位的 72 份脑组织及患者组织标本。通过比较 3 种方法的检测结果, 评价 dRIT 法的敏感性和特异性。同时分析 3 种方法的实验成本、技术要求等指标。讨论 dRIT 法的优势所在及适用范围。结果 3 种方法对所有标本的检测结果一致, dRIT 法具有与 DFA 法和 RT-PCR 法同等的敏感性和特异性。与其他两种方法相比, dRIT 法具有研究成本低、技术要求也较低的特点, 更适合于经费有限及技术力量较弱的实验室采用。结论 dRIT 法在狂犬病诊断中有良好的应用价值, 适于在我国基层 CDC 的狂犬病实验室检测工作中普及推广。

**【关键词】** 狂犬病; 诊断; 鉴别; 免疫组化技术

**The primary application of Direct rapid immunohistochemical test to rabies diagnosis in China** TAO Xiaoyan<sup>1</sup>, Michael Niezgoda<sup>2</sup>, Du Jialiang<sup>3</sup>, Li Hao<sup>3</sup>, Wang Xiaoyuan<sup>3</sup>, Huang Ying<sup>3</sup>, Jiang Peng<sup>3</sup>, Cao Lei<sup>3</sup>, Tang Qing<sup>3</sup>, Jiang Guoliang<sup>3</sup>, Department of Viral Hepatitis, Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 100025, China

Corresponding author: Tang Qing, E-mail: qiang04@china.com

**【Abstract】Objective** Evaluation of the direct rapid immunohistochemical test (dRIT) for laboratory surveillance of rabies. **Methods** 72 brain specimens of domestic dogs or patients collected from Guangdong, Guangxi, Hunan, Anhui, Jiangsu and Yunnan provinces were detected by conventional methods including Direct Fluorescent-antibody Assay (DFA) and Reverse Transcription Polymerase Chain Reaction (RT-PCR), and by dRIT which was newly developed in the Rabies Section of the Centers for Disease Control and Prevention in the United States. The sensitivity and specificity of dRIT were evaluated by compare of the three results. By analysis of the index including cost of experiment, technique requirement and so on, the advancement and applicability of dRIT were discussed. **Results** Compared with DFA and RT-PCR, dRIT was more applicable for laboratories with limited funds and weak techniques because of its lower cost needed and simpler techniques required while its sensitivity and specificity are equal to the other two methods. **Conclusion** dRIT is more valuable in rabies diagnosis and more applicable for extension and popularization in rabies laboratory surveillance in local CDC.

**【Key words】** Rabies; Diagnosis; differential; Immunohistochemistry

狂犬病是一种古老的兽共患传染病, 至今尚无有效治疗手段<sup>[1]</sup>。因此, 开展人及动物的狂犬病

监测是预防控制狂犬病的重要工作内容。其中, 实验室诊断方法的选择对于监测工作的顺利开展有重要作用。目前狂犬病实验室诊断主要应用世界卫生组织 (WHO) 推荐的 DFA 法<sup>[2]</sup>和 RT-PCR 法<sup>[3]</sup>。

近年狂犬病在我国流行严重, 病例主要分布于广大农村地区<sup>[4]</sup>。而 DFA 和 RT-PCR 法无论对仪器设备还是检测人员的技术经验均有较高要求<sup>[5,6]</sup>, 一般只有国家及省级实验室才具备相应的检测条件和

## Rabies Diagnosis for Developing Countries

Salome Dürr<sup>1</sup>, Service Naisengam<sup>2</sup>, Rolande Mindekem<sup>3</sup>, Colette Digumbye<sup>4</sup>, Michael Niezgoda<sup>5</sup>, Ivan Kuzmin<sup>6</sup>, Charles E. Rupprecht<sup>1</sup>, Jakob Zinsstag<sup>1\*</sup>

<sup>1</sup> Swiss Tropical Institute, Basel, Switzerland; <sup>2</sup> Laboratoire de Techniques Vétérinaires et Zootechniques de Fatick, N'Djamena, Tchad; <sup>3</sup> Centre de Support en Santé Internationale, N'Djamena, Tchad; <sup>4</sup> Center for Disease Control and Prevention, Atlanta, Georgia, United States of America

### Abstract

**Background:** Canine rabies is a neglected disease causing 55,000 human deaths worldwide per year, and 98% of all cases are transmitted by dog bites. In N'Djamena, the capital of Chad, rabies is endemic with an incidence of 1,717/1,000 dogs (95% CI: 1.45–1.98). The gold standard of rabies diagnosis is the direct immunofluorescent antibody (DFA) test, requiring a fluorescence microscope. The Centers for Disease Control and Prevention (CDC, Atlanta, United States of America) developed a histochemical test using low-cost light microscopy, the direct rapid immunohistochemical test (dRIT).

**Methodology/Principal Findings:** We evaluated the dRIT in the Chad National Veterinary Laboratory in N'Djamena by testing 35 fresh samples parallel with both the DFA and dRIT. Additional retests (n=68 in Chad, n=74 at CDC) by DFA and dRIT of stored samples enhanced the power of the evaluation. All samples were from dogs, cats, and in one case from a bat. The dRIT performed very well compared to DFA. We found a 100% agreement of the dRIT and DFA in fresh samples (n=35). Results of retesting at CDC and in Chad depended on the condition of samples. When the sample was in good condition (fresh brain tissue), we found simple Cohen's kappa coefficient related to the DFA diagnostic results in fresh tissue of 0.87 (95% CI: 0.63–1.1) up to 1. For poor quality samples, the kappa values were between 0.13 (95% CI: –0.15–0.40) and 0.48 (95% CI: 0.14–0.82). For samples stored in glycerol, dRIT results were more likely to agree with DFA testing in fresh samples than the DFA retesting.

**Conclusion/Significance:** The dRIT is as reliable a diagnostic method as the gold standard (DFA) for fresh samples. It has an advantage of requiring only light microscopy, which is 10 times less expensive than a fluorescence microscope. Reduced cost suggests high potential for making rabies diagnosis available in other cities and rural areas of Africa for large populations for which a capacity for diagnosis will contribute to rabies control.

**Citation:** Dürr S, Naisengam S, Mindekem R, Digumbye C, Niezgoda M, et al. (2008) Rabies Diagnosis for Developing Countries. PLoS Negl Trop Dis 2(3): e208. doi:10.1371/journal.pntd.002008

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**Competing Interests:** The authors have declared that no competing interests exist.

\* Email: jakob.zinsstag@univie.ac.at

### Introduction

Canine rabies is a zoonotic disease in most developing countries. Worldwide, approximately 55,000 (90% CI: 24,000–90,000) human deaths occur per year [1]. In N'Djamena, the capital of Chad, Kanari et al found a rabies incidence in the dog population of 1,717/1,000 (95% CI: 1.2–2.1) among unvaccinated dogs in 2001 [2]. In more than 99% of all human cases, the virus is transmitted by dog bite [3,4]. Administration of post exposure prophylaxis (PEP) immediately after the animal bite is essential to prevent productive infection, which is essentially fatal. FIP PEP requires human rabies immune globulin plus one dose of vaccine on days 0, 3, 7, 14 and 28 (even regions, recommendation World Health Organization [5]). In developing countries however, the vaccine is not always available and is expensive.

The WHO recommended gold standard of rabies diagnosis is the direct fluorescent antibody (DFA) test [4,5]. In Chad, the DFA test was established in 2000 in the Laboratoire de Recherches Vétérinaires et Zootechniques de Fatick (LRVZ) [2]. It is currently the only laboratory in the entire country where rabies

diagnosis with the DFA test is possible. Suspected rabies is reported from most areas of the country [6]. The need for a fluorescence microscope, which is expensive and difficult to maintain, limits the overall use of the DFA test in developing countries.

At the Centers for Disease Control and Prevention (CDC, USA), a direct rapid immunohistochemical test (dRIT) has been developed to detect rabies virus using an immunoperoxidase technique [7]. The dRIT uses highly concentrated and purified biotinylated anti-rabies monoclonal antibodies to rabies virus. After incubation with a streptavidin-peroxidase complex, the antibody complex is made visible with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen. The results can be read after less than one hour. Unlike the DFA test, the dRIT can be read after less than one hour. Unlike the DFA test, the dRIT requires all representative tissues examined in case. Using the test in the Senegal, Tanzania, Lesotho et al, found 100% sensitivity and specificity of the dRIT compared to the gold standard DFA test [8]. The preservation of rabies samples with glycerol buffer (50% glycerol solution in 0.01 M phosphate-buffered saline), as it was used in the mentioned study [8], is an

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PLOS Neglected Tropical Diseases



## Comparison of Biotinylated Monoclonal and Polyclonal Antibodies in an Evaluation of a Direct Rapid Immunohistochemical Test for the Routine Diagnosis of Rabies in Southern Africa

Andre Coetzee<sup>1</sup>, Claude T. Sabeta<sup>2</sup>, Wanda Markotter<sup>3</sup>, Charles E. Rupprecht<sup>3</sup>, Louis H. Nel<sup>1\*</sup>

<sup>1</sup> Department of Microbiology and Parasitology, University of Pretoria, Ga-rosburg, South Africa, <sup>2</sup> Agricultural Research Council-Onderstepoort Veterinary Institute, Rabies Division, Ga-rosburg, South Africa, <sup>3</sup> Ross University School of Veterinary Medicine, Boxtown, St. Kitts, West India

### Abstract

The major etiological agent of rabies, rabies virus (RVB), accounts for tens of thousands of human deaths per annum. The majority of these deaths are associated with rabies cycles in dogs in resource-limited countries of Africa and Asia. Although routine rabies diagnosis plays an integral role in disease surveillance and management, the application of the currently recommended direct fluorescent antibody (DFA) test in countries on the African and Asian continents remains quite limited. A novel diagnostic assay, the direct rapid immunohistochemical test (dRIT), has been reported to have a diagnostic sensitivity and specificity equal to that of the DFA test while offering advantages in cost, time and interpretation. Prior studies used the dRIT utilized monoclonal antibody (MAb) cocktails. The objective of this study was to test the hypothesis that a biotinylated polyclonal antibody (PAb) preparation, applied in the dRIT protocol, would yield equal or improved results compared to the use of dRIT with MAbs. We also wanted to compare the PAb dRIT with the DFA test, utilizing the same PAb preparation with a fluorescent rabbit. The PAb dRIT had a diagnostic sensitivity and specificity of 100%, which was shown to be marginally higher than the diagnostic efficacy observed for the PAb DFA test. The classical dRIT, relying on two-biotinylated MAbs, was applied to the same panel of samples and a reduced diagnostic sensitivity (83.50% and 90.78% respectively) was observed. Antigenic typing of the false-negative samples indicated all of these to be monophase RVB variants. Our results provided evidence that a dRIT with alternative antibody preparations, conjugated to a biotin moiety, has a diagnostic efficacy equal to that of a DFA relying on the same antibody and that the antibody preparation should be optimized for virus variants specific to the geographical area of focus.

**Citation:** Coetzee A, Sabeta CT, Markotter W, Rupprecht CE, Nel LH (2014) Comparison of Biotinylated Monoclonal and Polyclonal Antibodies in an Evaluation of a Direct Rapid Immunohistochemical Test for the Routine Diagnosis of Rabies in Southern Africa. PLoS Negl Trop Dis 8(2): e8108. doi:10.1371/journal.pntd.0081088

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**Competing Interests:** The authors have declared that no competing interests exist.

\* Email: lohn.nel@up.ac.za

### Introduction

Rabies is a neglected zoonosis that is responsible for the death of tens of thousands of people per annum [1]. The majority of human rabies deaths are associated with canine rabies in resource-limited countries. Rabies is caused by multiple lyssaviruses (Genus: *Lyssavirus*, Family: *Rhabdoviridae*), of which the prototype is rabies virus (RVB). While RVB is most important from a global disease perspective, there are more than 12 other lyssavirus species, most of which have been associated with infrequent cases of human rabies [2,3]. Although classical rabies has the highest known case-fatality rate of any infectious disease, and is preventable by means of effective pre- and post-exposure prophylaxis, the disease is still widespread throughout developing countries on the African and Asian continents [1,4–7]. The process of post-exposure diagnostic confirmation of rabies plays a crucial role in general disease surveillance and is also involved in disease management programs for animal populations (e.g., identifying disease outbreaks within geographical regions where dog vaccination campaigns are being implemented), as well as in risk assessment for consideration of human prophylaxis.

In the case of resource-limited developing countries, where limited or no diagnostic confirmation is undertaken, very little rabies data are reported to relevant authorities. In some instances it has also been found that even though limited diagnosis may occur, the diagnostic results are not reported to the relevant authorities at all. This appears to be due to various logistical reasons, such as a lack of record keeping, limited communication, etc. [8]. As a result of the under estimation of the disease in animal populations, developing countries typically give little or no support and rabies remains of low political priority [1,9]. The documentation of the disease burden is thus dependent on proper surveillance and diagnostic activities to break this cycle of neglect.

The gold standard assay for rabies diagnosis is the direct fluorescent antibody (DFA) test [9,10], but proper application of this method in much of the developing world remains limited. This is due in part to a lack of reliable infrastructure (power supply, access to running water and good quality water disposal), and



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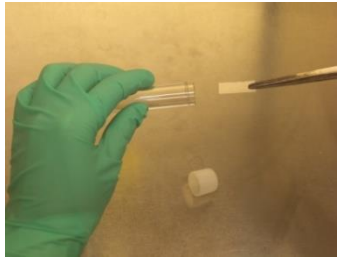
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OIE SEA Sub-Regional Virtual Workshop on OIE international standards supporting self-declaration of freedom from rabies, and endorsement of official control programme, 6-8 July 2021



## 1.3.2 Virus isolation

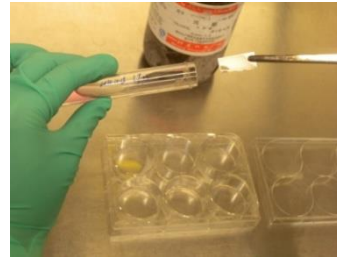
### i) Rabies Tissue Culture Isolation Test (RTCIT)



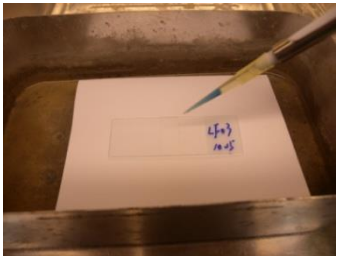
Place a coverslip into the inclined plane



Add brain suspension sample to the cell



Fix the plane



Add antibody conjugate

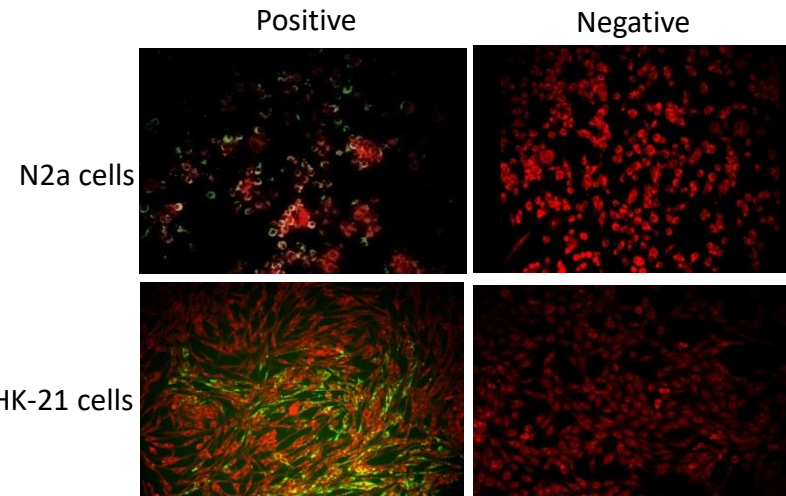


Incubation



Observation

- OIE and WHO standard
- high sensitivity and specificity
- used for virus detection and isolation
- National standard of China: GB/T18639-2002
- disadvantage: time consuming, BSL3 facility
- N2A and BHK-21 cell lines



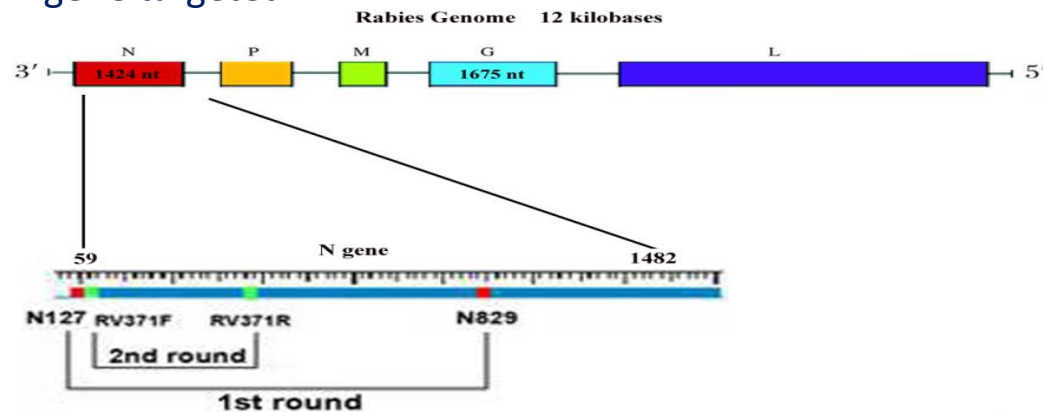
ii) Mouse inoculation test (MIT) is less used due to animal welfare.



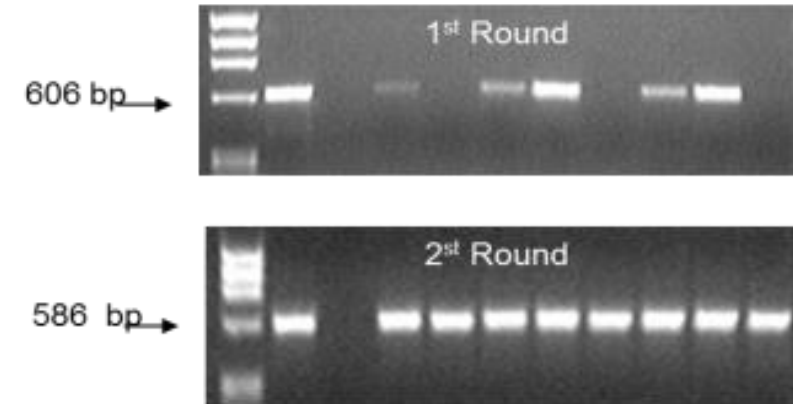
## 1.3.3 Conventional RT-PCR

- OIE and WHO recommended
- high sensitivity and specificity
- Genotyping: phylogenetic analysis
- Pan-lyssavirus
- Disadvantage: unable to differentiate; false positive or false negative; very stringent quality control (partition of room).

N gene targeted



i) OIE manual



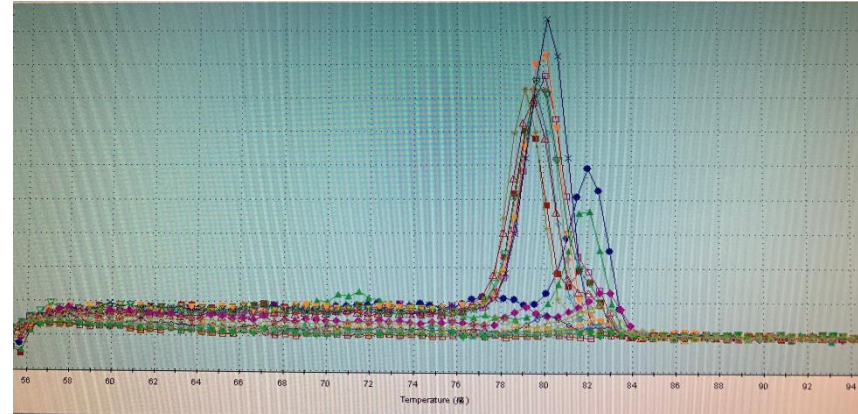
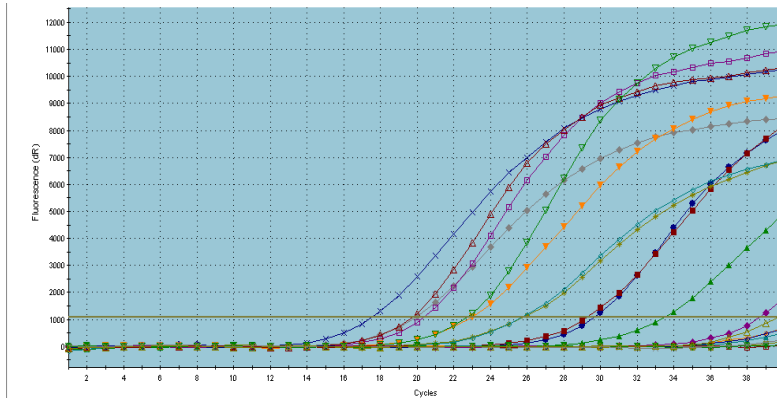
ii) Chinese RL method



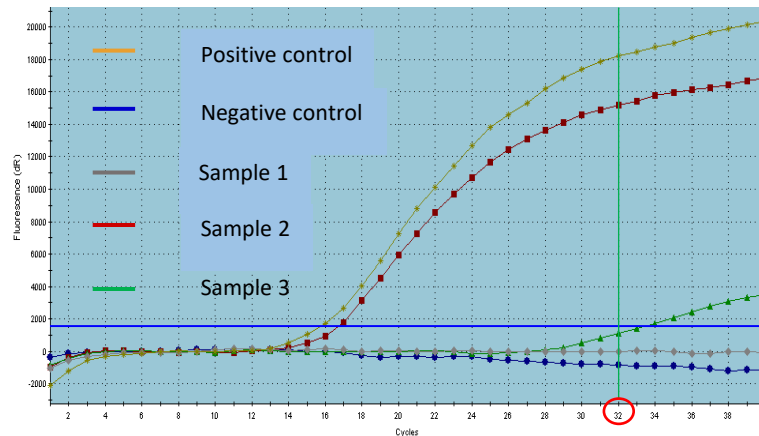


## 1.3.4 Real time RT-PCR

i) OIE manual: SYBR green method, **Pan-lyssavirus**



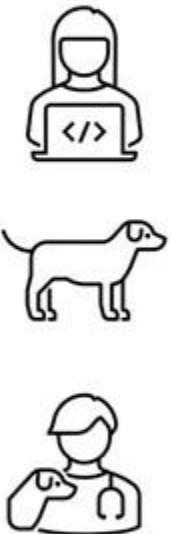
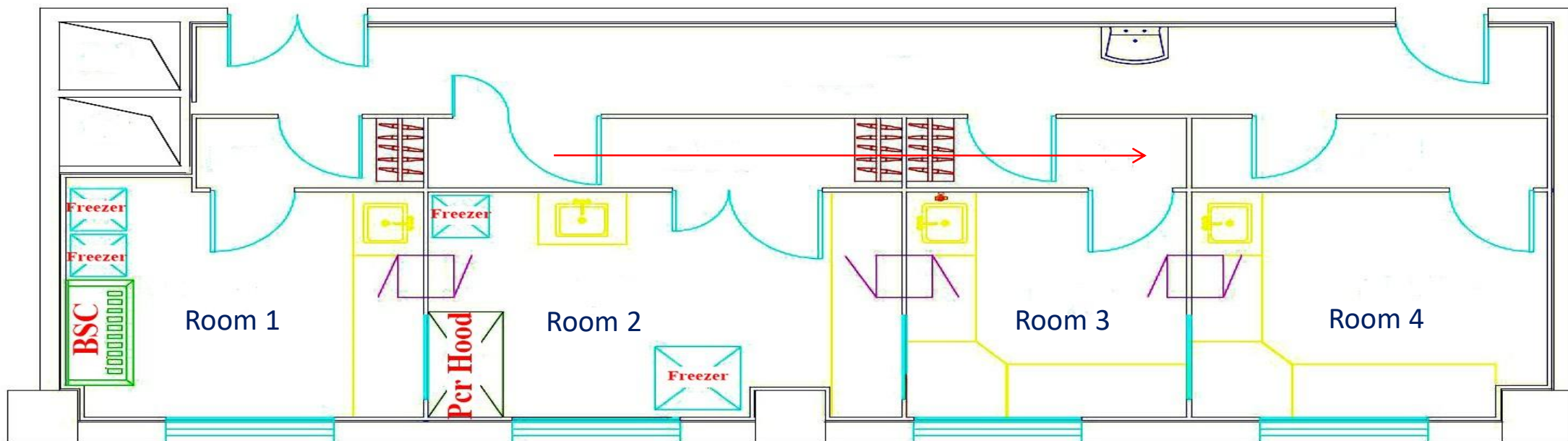
ii) Chinese RL method: Taq man method, **RABV-specific**



- Positive sample :  $0 < Ct < 32$  (sample 2)
- Negative sample: No Ct (sample 1)
- If  $Ct > 32$  : Repeat assay for verifying (sample 3)

# Room partition for PCR: Four-room principle

- Room 1: Reagent storage and pre-mixture prep
- Room 2: Prep of viral nucleic acid and PCR reaction
- Room 3: PCR
- Room 4: Gel running and documentation



# Test of decomposed brain tissue

## Comparison of 4 tests in detection of two decomposed samples

Day post decomposition	FAT*	RT-nPCR*	Tagman RT-PCR*	MIT*
1	+/+	+/+	+/+	-/-
2	+/+	+/+	+/+	-/-
3	+/+	+/+	+/+	-/-
4	+/+	+/+	+/+	-/-
5	+/+	+/+	+/+	-/-
6	+/+	+/+	+/+	-/-
7	+/-	+/+	+/+	-/-
8	-/-	+/+	+/+	-/-
9	-/-	+/+	+/+	-/-
10	-/-	+/+	+/+	-/-
11	-/-	+/+	+/+	-/-
12	-/-	+/+	+/+	-/-
13	-/-	+/+	+/+	-/-
14	-/-	+/+	+/+	-/-
15	-/-	+/+	+/+	-/-
16	-/-	+/+	+/+	-/-
17	-/-	+/+	+/+	-/-

Note: "+"positive; "-"negative \*TJ105 strain/CQQJ-09 strain



## 2. Serological Test

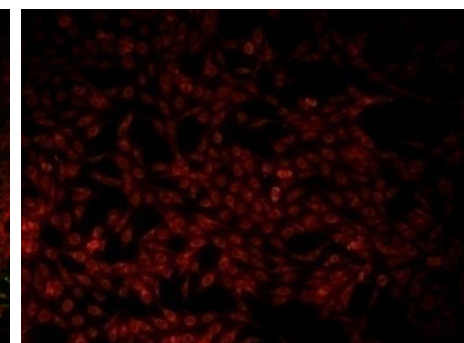
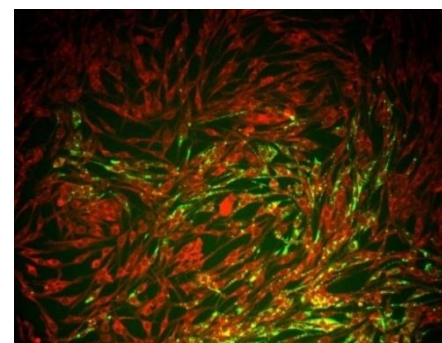
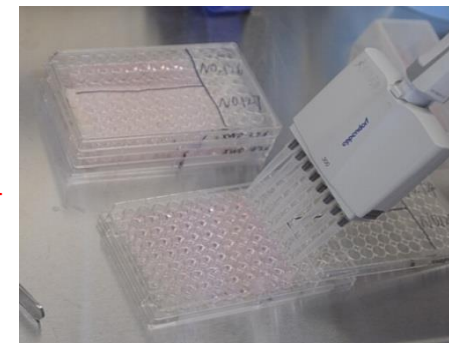
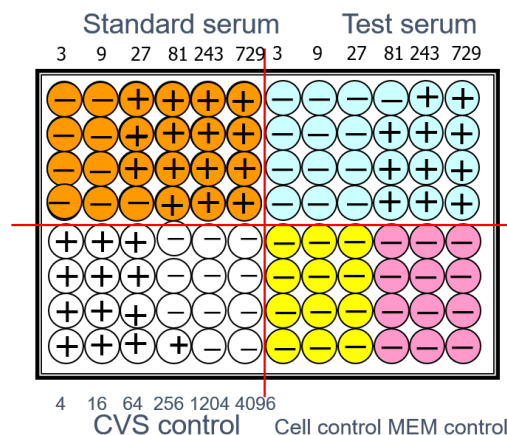
Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
VN	n/a	+++	+++	n/a	n/a	+++
ELISA	n/a	n/a	+++	n/a	n/a	+++





## 2.1 Fluorescent Antibody Virus Neutralization (FAVN)

- OIE and WHO standard
- Prescribed test for international trade or travel of live animals
- Quantification of antibody level (0.5 IU/ml)
- Most widely used in the world
- Requisite for professional rabies laboratory
- Disadvantage: time-and-labor-consuming, BSL2, high cost.



- Formula to convert the log D<sub>50</sub> value in IU/ml titre:

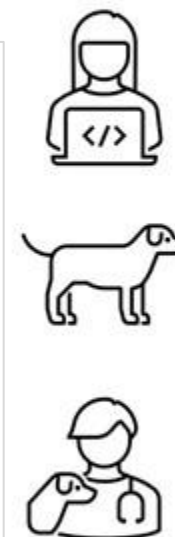
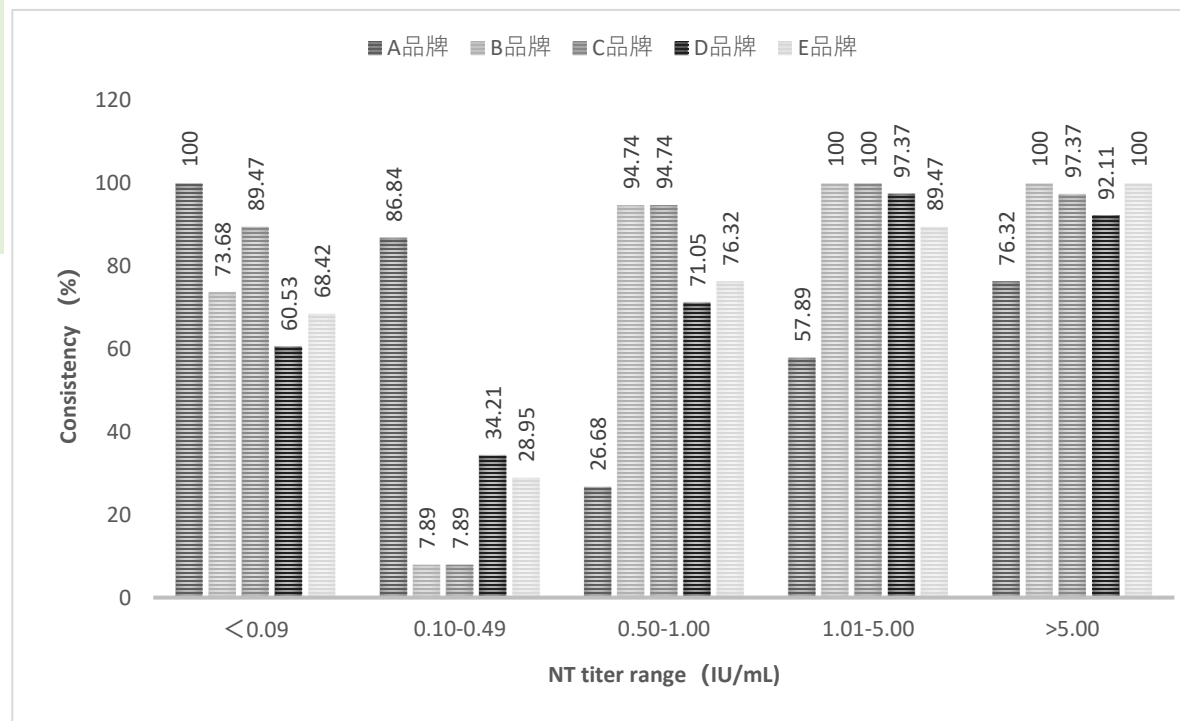
$$\text{Serum titre (IU/ml)} = \frac{[(10^{\text{serum log D}_{50} \text{ value}}) \times \text{theoretical titre of OIE serum 0.5 IU/ml}]}{(10^{\text{log D}_{50} \text{ of OIE serum 0.5 IU/ml}})}$$

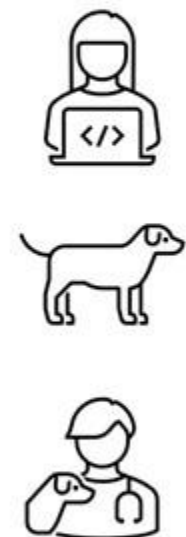
## 2.2 Ab ELISA

- OIE recommended
- Suitable to detect the herd immunity, not for titration of individual animals
- Commercialized
- Advantage: rapid, no live virus, large scale screening
- Disadvantage: not well correlates with VN, **not for international trade or travel of live animals.**

Brand	Sensitivity	Specificity
A	52.63% (60/114)	93.42% (71/76)
B	98.25% (112/114)	40.79% (31/76)
C	97.37% (111/114)	48.68% (37/76)
D	86.84% (99/114)	47.37% (36/76)
E	88.60% (101/114)	48.68% (37/76)

Comparison of 5 commercial kits with FAVN





Thank you for your attention!